

Novel customisable phases for micro solid-phase extraction and automated biological sample preparation

by Karen Duong

Thesis submitted in fulfilment of the requirements for
the degree of

Doctor of Philosophy

under the supervision of Distinguished Professor Philip
Doble, Dr David Bishop and Dr Raquel Gonzalez de Vega.

University of Technology Sydney
Faculty of Science

August 2021

Certificate of authorship and originality

I, Karen Duong declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Mathematical and Physical Sciences at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

This research is supported by the Australian Government Research Training Program.

Signature:

Production Note:

Signature removed prior to publication.

Date: 30th August 2021

Acknowledgements

This thesis wouldn't have been possible without the help and support of many people.

I would like to thank my supervisors, Distinguished Professor Philip Doble, Dr David Bishop and Dr Raquel Gonzalez de Vega for their expertise, great advice, time, and emotional support. They have been instrumental to the success of this project. Dr Raquel Gonzalez de Vega played a significant role and her contributions to this work are greatly appreciated which involved help in performing the final experiments involving the complete automated workflow work with BSA and human serum samples.

Thank you to Peter Dawes, Andrew Minett, Reno Cerra and Mark Wardle at Eprep for the opportunity to work on this project. They have provided countless hours of support. Furthermore, I'd like to express my gratitude to UTS and Eprep for the opportunity to be involved in the Industry Doctorate Program. This targeted PhD program has provided me with invaluable skills and experiences for my career and personal development.

Thank you to Dr David Clases for his assistance with my research project, helping me run instruments, and for offering great support and ideas. I would also like to thank Dr Matt Padula and Joel Steele for their help with proteomics and running samples. Thank you to Dr Ronald Shimmon and Dr Dayanne Bordin for providing great technical services.

I'd like to thank the analytical research group, Mika Westerhausen, Matthew Diplock, Phuc Nguyen, Prashina Singh, Natasha Benson-Jeffrey, Sarah Meyer, Thomas Lockwood and Brooke Mansell for their comradery, support and good times. Would like to thank my peers, Minh Nguyen for being a good lunch buddy and always bringing the best snacks, and Jacqueline Loyola-Echeverria for providing moral support. Thank you to Susan Shimmon and Mahmoud El Safadi for the great times we had in laboratory.

I want to give a huge thanks to my family who encouraged me to finish. They have showed me never-ending love and support, and to them I am so grateful. Thank you to all my friends for your support and words of encouragement. Particularly, a massive thanks to Hue for being there for me through the highs and lows. Finally, I'd like to give my utmost thanks to the LORD God for his providence and guidance over all things.

Abstract

Protein biomarkers play an important role in clinical settings as they serve as measurable indicators for normal or abnormal biological processes. They aid in accelerated disease identification, diagnosis, prognosis, and response to treatments. Recently, mass spectrometry (MS) assays have been introduced as an alternate to the conventionally used immunoassays. MS provides accurate and precise quantification due to its high sensitivity and specificity. However, major challenges lie with sample preparation involving lengthy workflows, limited automation, and challenges related to highly complex biological samples where several techniques are applied.

This thesis aims to improve sample preparation techniques to provide an accurate, rapid, and automated method for protein biomarker quantification using micro-solid-phase extraction (μ SPE) technology through the development of a micro-immobilised-enzyme reactor (IMER) and a μ SPE immunoaffinity cartridge to alleviate sample preparation bottlenecks caused by conventional ~18-hour digestions.

A novel and customisable material was prepared for bio-ligand immobilisation. A hybrid inorganic-organic material using silica modified with carboxymethylated dextran (CMD) was prepared. This material was packed into μ SPE cartridges and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) coupling was exploited to immobilise enzymes and antibodies in-situ using a programmable syringe driver allowing for the development of an automated workflow.

Reproducible trypsin digestion was observed using high performance liquid chromatography (HPLC) through the cleavage of N- α -Benzoyl-L-arginine ethyl ester (BAEE). Three model proteins (bovine serum albumin, cytochrome c and thyroglobulin) and a human serum sample were analysed, and compared to conventional in-solution digestion by employing liquid chromatography orbitrap mass spectrometry (LC-OT-MS) and liquid chromatography quadrupole time of flight mass spectrometry (LC-QToF-MS). The IMER facilitated protein digestion within 10 minutes at room temperature and overall, observed lower sequence coverages and number of identified proteins compared to the conventional in solution digestion method at 37°C.

In the same manner as trypsin, anti-BSA was immobilised and BSA isolation was confirmed using size exclusion chromatography hyphenated to triple quadrupole inductively coupled plasma mass spectrometry (SEC-ICP-MS/MS). Whilst immobilisation of anti-BSA was achieved, challenges with low level protein detection were observed.

The immunoaffinity and trypsin μ SPE cartridge were combined into an automated workflow for protein pre-concentration and digestion. Using BSA as the model protein, BSA standards and BSA spiked into human serum samples were subjected to the workflow. BSA extraction and digestion was achieved, however, the complex human serum matrix negatively impacted the BSA isolation compared to neat standards. Further investigation and optimisation of the workflow must be performed.

List of Publications and Presentations

Journal Publications

Immunoaffinity extraction followed by enzymatic digestion for the isolation and identification of proteins employing automated μ SPE reactors and mass spectrometry (Submitted to Analytical and Bioanalytical Chemistry).

Application Notes

Karen Duong, Simin Maleknia, Andrew Minett, David Bishop, Philip Doble, " μ SPEed-Cxyl microreactor cartridges: Trypsin cartridges for digests of bovine serum albumin (BSA)", ePrep Application Note, 2019.

Karen Duong, Philip Doble, " μ SPEed recovery curve with 4 nitrotoluene", ePrep Application Note, 2015.

Conference Poster Presentations

Karen Duong, Raquel Gonzalez de Vega, Andrew Minett, David Bishop, Philip Doble, "*Immunoaffinity and enzymatic reactor micro-solid-phase extraction cartridges for rapid protein isolation and digest*", American Society for Mass Spectrometry Conference, 2019, Atlanta, Georgia, USA.

Karen Duong, Simin Maleknia, Andrew Minett, David Bishop, Philip Doble, "*Immobilised enzymes on micro-solid-phase extraction cartridges for automated protein digestion*", American Society for Mass Spectrometry Conference, 2018, San Diego, California, USA.

Karen Duong, Simin Maleknia, Andrew Minett, David Bishop, Philip Doble, "*Customisable micro-solid-phase extraction for selective binding in biological sample preparation*", The Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, 2018, Orlando, Florida, USA.

Karen Duong, Simin Maleknia, Andrew Minett, David Bishop, Philip Doble, "*Immobilised enzymes on customisable micro-solid-phase extraction cartridges for proteolytic digests in minutes*", Lorne Proteomics Symposium, 2018, Lorne, Melbourne, Australia.

Table of Contents

Certificate of authorship and originality	i
Acknowledgements.....	ii
Abstract.....	iii
List of Publications and Presentations.....	v
Table of Contents.....	vi
List of Abbreviations and Acronyms	viii
List of Figures	xii
List of Tables.....	xvi
Chapter 1 Introduction: Protein biomarker quantification for clinical proteomics.....	1
1.1 Biomarkers for Clinical Proteomics.....	2
1.1.1 Protein Biomarker Quantification	3
1.1.2 Molecular mass spectrometry for the analysis and determination of potential biomarkers.....	5
1.1.3 Elemental mass spectrometry for the analysis and determination of potential biomarkers.....	7
1.1.4 Sample preparation for biomarker analysis	10
1.2 Solid Phase Extraction	15
1.2.1 Miniaturisation, automation, and progression of SPE methods	17
1.2.2 Customisable material for targeted sample preparation.....	20
1.3 Project Aims	30
Chapter 2 Customisable support material for biologically selective and automated sample preparation using the ePrep μSPE format.....	33
2.1 Introduction.....	34
2.2 Experimental.....	37
2.2.1 Chemicals and Consumables.....	37
2.2.2 μ SPE method	37

2.2.3	Development of customisable material for μ SPE cartridges	37
2.3	Results and Discussion	41
2.4	Conclusion	50
Chapter 3	Development of rapid and automated μSPE sample preparation workflows for protein biomarkers	51
3.1	Introduction.....	52
3.2	Experimental.....	55
3.2.1	Chemicals and Consumables.....	55
3.2.2	μ SPE sample preparation workflow development	55
3.3	Development of the IMER μ SPE cartridge	59
3.3.1	Instrumentation and parameters for IMER μ SPE cartridge	59
3.3.2	Sample preparation for protein digestion using model proteins and human serum sample	62
3.3.3	Results and Discussion.....	63
3.4	Development of the immunoaffinity μ SPE cartridge.....	78
3.4.1	Instrumentation and parameters for immunoaffinity μ SPE cartridge.....	78
3.4.2	Exogenous labelling of proteins.....	79
3.4.3	Antibody immobilisation and Ab-Ag complex formation	80
3.4.4	Results and Discussion.....	82
3.5	Combination of the immunoaffinity and IMER μ SPE cartridges for an automated workflow.....	89
3.5.1	μ SPE workflow.....	89
3.5.2	Results and Discussion.....	89
3.6	Conclusion	91
Chapter 4	: Overall Conclusions and Future Work.....	93
References		98

List of Abbreviations and Acronyms

Ab-Ag: antibody-antigen complex

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

ACN: acetonitrile

AGC: automated gain control

APS: aminopropyl silica

ATR: attenuated total reflectance

BA: N_{α} -Benzoyl-L-arginine

BAEE: N_{α} -benzoyl-L-arginine ethyl ester

BCA: bicinchoninic acid

BEC: background equivalent concentration

BIN: barrel insert and needle

BSA: bovine serum albumin

CID: collision induced dissociation

CMD: carboxymethylated dextran

Cyt c: cytochrome c

CV: coefficient of variation

Da: dalton

DMSPE: dispersive micro-solid-phase extraction

DNA: deoxyribonucleic acid

DSS: disuccinimidyl suberate

DTT: dithiothreitol

EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide

ELISA: enzyme-linked immunosorbent assays

ESI: electrospray ionisation

F_{ab}: fragment antigen binding

F_c: fragment crystallisable

FDR: false discovery rate

FI: flow injection

FTIR: Fourier-transform infrared spectroscopy

GC: gas chromatography

HAPs: high abundant proteins

HPLC: high performance liquid chromatography

HRP: horseradish peroxidase

IA: iodoacetamide

IASPE: immunoaffinity-based solid-phase extraction

ICP-MS: inductively coupled plasma mass spectrometry

ID: inside diameter

Ig: immunoglobulin

IMAC: immobilised metal affinity chromatograph

IMER: immobilised enzyme reactor

ITSP: instrument top sample preparation

KHP: potassium hydrogen phthalate

LAPs: low abundant proteins

LC: liquid chromatography

LAC: lectin affinity chromatography

LLE: liquid-liquid extraction

mAb: monoclonal antibody

MALDI: matrix assisted laser desorption/ionisation

MEPS: microextraction by packed sorbents

MES: 2-(*N*-morpholino)ethanesulfonic acid

MIPs: molecularly imprinted polymers

MISPE: molecularly imprinted solid-phase extraction

MOAC: metal oxide affinity chromatography

MRM: multiple reaction monitoring

MS: mass spectrometry

MS/MS: tandem mass spectrometry

MSPE: magnetic solid-phase extraction

MW: molecular weight

***m/z*:** mass-to-charge ratio

NHS: *N*-hydroxysuccinimide

OAI: oriented antibody immobilisation

OD: outside diameter

OT: orbitrap

pAb: polyclonal antibody

PBS: phosphate buffer saline

PEG: poly(ethylene glycol)

***pI*:** isoelectric point

PIT: pre-immobilised trypsin

PPT: protein precipitation

PRM: parallel reaction monitoring

Q1, Q2, Q3: quadrupole 1, 2 and 3

Q-OT: quadrupole-orbitrap

QQQ: triple quadrupole

RAI: random antibody immobilisation

RNA: ribonucleic acid

SBSE: stir-bar sorptive extraction

SEC: size exclusion chromatography

SPE: solid-phase extraction

SPME: solid-phase microextraction

SPR: surface plasmon resonance

SRM: selected reaction monitoring

TCEP: tris(2-carboxyethyl)phosphine hydrochloride

TPCK: L-1-tosylamide-2-phenylethyl chloromethyl ketone

TIC: total ion chromatogram

ToF: time-of-flight

UV: ultraviolet

UV-Vis: ultraviolet-visible

List of Figures

Figure 1.1: Illustration of a QQQ mass spectrometer with mass filters (Q1, Q3), and collision cell (Q2) [58].	6
Figure 1.2: Protein interactions with porous stationary phases used in SEC [77].	8
Figure 1.3: MS/MS mode using an oxygen cell for mass shifting of ^{32}S (m/z 32) to $^{32}\text{S}^{16}\text{O}$ (m/z 48) [87].	9
Figure 1.4: Sample preparation workflow for targeted quantification of protein biomarkers [101].	10
Figure 1.5: Bind-elute strategy for SPE [170].	17
Figure 1.6: Non-specific interaction between silica surface and proteins. A) electrostatic interaction between negatively charged surface and positively charged residues on the protein, and B-D) protein deformation due to weak internal cohesion resulting in deformation and hydrophobic interaction with the silica surface [254].	22
Figure 1.7: Immobilisation strategies used for biomolecules (E) [148].	24
Figure 1.8: A) Avidin-biotin complex with four binding sites, and B) Bicyclic structure of biotin with a carboxylic acid side chain that can be modified [283].	26
Figure 1.9: Antibody immobilisation through intermediate proteins: protein A or G [280].	26
Figure 1.10: Homo- and hetero- bifunctional crosslinkers with reactive groups held by a spacer arm [291, 292].	28
Figure 1.11: EDC activation of a carboxylic group to form an unstable o-acylisourea intermediate that when reacted with an amine compound, forms an amide bond between the carboxylate compound and amine compound [295].	29
Figure 1.12: Primary amine reacting with the stable NHS ester to form an amide bond [295].	29
Figure 1.13: -SH groups obtained through disulfide bridge reduction [305].	30
Figure 2.1: digiVOL® syringe driver used for method development and the press-fit μSPEed cartridge onto a syringe. The μSPEed offers two flow paths where solutions are aspirated through the one-way valve and dispensed onto the sorbent packing material [314].	36
Figure 2.2: ePrep® Sample Preparation Workstation showing sample trays and liquid dispensing syringes [314].	36

Figure 2.3: Methods for non-covalent and covalent immobilisation of HRP onto silica-CMD cartridges.	40
Figure 2.4: FTIR spectra obtained for starting materials a) APS, b) CMD (red), and the synthesised material c) silica-CMD.....	42
Figure 2.5: Conductometric titration of the blank control with no silica-CMD material.....	43
Figure 2.6: A) Conductometric titration of silica-CMD prepared from 120 Å material with the first derivatives for the B) starting and C) end point of the neutralisation of -COOH groups on the silica-CMD.....	45
Figure 2.7: Conductometric titration of silica-CMD prepared from 300 Å material.....	47
Figure 2.8: FTIR of 1000 Å APS material following attempted CMD coating.....	47
Figure 2.9: Absorbance of ABTS radical cation at 620 nm using the Thermo Fisher Multiskan Ascent 96/384 Plate Reader to compare between non-covalent and covalent immobilisation of HRP onto silica-CMD material.	49
Figure 3.1: Automated μ SPEed biomarker workflow for both protein immunoaffinity isolation and digestion.....	58
Figure 3.2: Observing the presence of BAEE (4 min) hydrolysis to BA (2 min) by excess non-covalently bound trypsin in collected salt wash buffer aliquots. A) Chromatogram shows BAEE hydrolysis following the first salt wash buffer wash and B) a chromatogram showing no presence of trypsin in subsequent washes.....	64
Figure 3.3: Comparison of two BSA digests (5 μ g protein load) using different CaCl_2 concentrations. Aliquot A1) TIC for BSA digest in running buffer containing 1000 μM CaCl_2 and Aliquot A2) TIC for elution buffer aliquot following the 1000 μM CaCl_2 running buffer digest using 25 mM Tris, 10 mM CaCl_2 and 10% ACN showing minimal peptide adsorption. Aliquot B1) TIC for BSA digest in running buffer containing 500 μM CaCl_2 and the Aliquot B2) TIC for elution buffer aliquot following the 500 μM CaCl_2 running buffer using 25 mM Tris, 10 mM CaCl_2 and 10% ACN showing peptide adsorption. C) TIC of 50 ng/ μL BSA standard that was not digested.....	66
Figure 3.4: Cyt c adsorption onto a) -COOH glycine end-capped as observed by the red appearance, and the absence of cyt c adsorption onto b) -OH ethanolamine end-capped silica-CMD.....	67

Figure 3.5: HPLC-UV chromatogram at 254 nm for BAEE (4 min) digestion to BA (2 min) for a) 2-minute digest and b) 5-minute digest.....	68
Figure 3.6: TICs of cartridge digests of Cyt c with varying protein amounts. A) A 5-minute digest of 10 µg of Cyt c showing remaining undigested protein whilst B) a 5-minute digest of 1 µg Cyt c shows no undigested protein.	69
Figure 3.7: TICs of cartridge digests of BSA with varying protein amounts. A) A 10-minute digest of 5 µg BSA showing undigested protein whilst B) a 10-minute digest of 1 µg BSA with minimal protein remaining.	70
Figure 3.8: A) and B) show TIC of BSA digest using two trypsin cartridges where 15 pmol of BSA was loaded with an incubation time of 10 minutes. Analysis was performed using the LC-OT-MS and the resulting injected peptides from 375 fmol of digested protein. C) TIC of a blank trypsin cartridge digest where running buffer was used.	71
Figure 3.9: Two BSA cartridge digests (top being 61% sequence coverage and bottom 59%), showing high overlap of protein sequence coverage.....	72
Figure 3.10: Standard calibration curve constructed for BAEE after analysis with HPLC-UV. ..	73
Figure 3.11: Number of identified peptides using IMER and in-solution digests for BSA, Cyt c and Tg, and the number of peptides observed across both methods.....	75
Figure 3.12: Protein sequence coverage for the A) overnight and B) cartridge digest of BSA, highlighting the amino acids that were unique to each respective sequence coverage and not observed in the other.	76
Figure 3.13: Protein sequence coverage for the A) overnight and B) cartridge digest of Cyt c, highlighting the amino acids that were unique to each respective sequence coverage and not observed in the other.	76
Figure 3.14: Protein sequence coverage for the A) overnight and B) cartridge digest of Tg, highlighting the amino acids that were unique to each respective sequence coverage and not observed in the other.	77
Figure 3.15: Example of Maxpar™ labelling through maleimide linkages on desired biomolecule containing free -SH groups [353].	80
Figure 3.16: A) Covalent immobilisation of anti-BSA onto silica-CMD and visualisation with mouse IgG HRP polymer antibody and B) covalent immobilisation of BSA onto silica-CMD for	

complex formation with anti-BSA, and visualisation with mouse IgG HRP polymer antibody.	81
Figure 3.17: Visualisation of HRP activity with mouse IgG HRP polymer and ABTS substrate for the confirmation of A) covalent immobilisation of anti-BSA onto silica-CMD as well as the B) BSA immobilisation followed by complex formation between anti-BSA and BSA, and C) and D) as their respective blank controls where no covalent immobilisation was performed using EDC/NHS.	83
Figure 3.18: Protein ladder calibration used to calculate MW of the biomolecule using the retention time using the Agilent Bio SEC-3 column.	84
Figure 3.19: SEC-ICP-MS/MS analysis using the Agilent Bio SEC-3 column for BSA (black), anti-BSA (red), and Ab-Ag complex (orange) with corresponding MW calculations using the protein ladder calibration.	85
Figure 3.20: Chromatographic separation of BSA (S signal, red) and labelled BSA-Er (Er signal, black) by SEC-ICP-MS/MS analysis using the Waters ACQUITY UPLC Protein BEH SEC.	85
Figure 3.21: Chromatograms obtained from the analysis of salt wash buffer aliquots following BSA sample loading onto an ePrep cartridge and analysed using FI-ICP-MS/MS for detection of $^{32}\text{S}^{16}\text{O}$	87
Figure 3.22: Chromatograms obtained from the analysis of salt wash buffer aliquots following BSA-Er sample loading onto an ePrep cartridge and analysed using FI-ICP-MS/MS for detection of ^{166}Er	87
Figure 3.23: Analysis of eluted sample following immunoaffinity extraction of BSA with an anti-BSA ePrep cartridge. Sample was analysed using SEC-ICP-MS/MS for detection of $^{32}\text{S}^{16}\text{O}$	88
Figure 3.24: Analysis of eluted sample following immunoaffinity extraction of BSA with an anti-BSA ePrep cartridge. Sample was analysed using FI-ICP-MS/MS for detection of $^{32}\text{S}^{16}\text{O}$	88
Figure 3.25: BSA protein coverage (26%) obtained by LC-OT-MS of the spiked human sample after protein isolation followed by tryptic digestion.	90

List of Tables

Table 1.1: Enzyme immobilisation techniques and their advantages and disadvantages [148].	24
Table 2.1: Calculated mmol of -COOH groups per gram of customised silica-CMD material for n=7 batches.	45
Table 2.2: Relationship between pore size and surface area for APS, and the resulting mmol of -COOH groups from the synthesis of silica-CMD [327].	47
Table 3.1: HPLC conditions for analysis of the trypsin digest of BAEE.	59
Table 3.2: Analysis conditions for protein digestion on LC-MS instruments.	61
Table 3.3: Two cartridge digestion methods with differing running buffer salt concentrations followed by an elution buffer to observe for any peptide adsorption to the cartridge as a result. All aliquots were analysed by LC-QToF-MS.	65
Table 3.4: Effect of flow rate on BAEE digest whilst ensuring the total incubation for both digests remain the same.	69
Table 3.5: Calculated BAEE concentration following a 2-minute incubation with six trypsin cartridges.	73
Table 3.6: BSA sequence coverages for μ SPE cartridge digest and BSA MS overnight in-solution digest.	76
Table 3.7: Typical operating conditions for the 8900 ICP-MS/MS.	78
Table 3.8: SEC calibration for the protein ladder standard used to calculate sample.	84
Table 3.9: Obtained BSA protein coverage after immunoaffinity extraction followed by tryptic digestion using three affinity cartridge twice.	90